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## Evaluation of Antimicrobial Efficacy of Ethanol Extracts of Fresh *Citrus sinensis* (Sweet Orange) Seeds against Selected Bacterial Strains

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**ABSTRACT:** The antibacterial activities of fresh *Citrus sinensis* seed oil and non-oil extract are investigated in this study. Gram positive (*Staphylococcus aureus* and *Enterococcus faecalis*) and Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella spp.*) bacterial strains are used in this study and antibacterial activity is estimated using the agar well diffusion method. Zones of inhibition were measured and the minimum inhibitory concentration and minimum bacteriostatic concentrations were determined. The results show that the non-oil extract had better antibacterial activities against *Staphylococcus aureus*, *Enterococcus faecalis* and *Escherichia coli* compared to the oil extract (zones of inhibition at 200 µg/ml of 12 mm, 8 mm and 2 mm respectively for the non-oil extract; 4 mm, 6 mm, 0 mm respectively for the seed oil). The seed oil however had better antibacterial activity compared to the non-oil extract in *Salmonella spp.* (zones of inhibition at 200 µg/ml of 8 mm for the seed oil compared to 4 mm for the non-oil extract). *Pseudomonas aeruginosa* showed similar susceptibility to both extracts at both concentrations studied (zones of inhibition of 4 mm and 2 mm for both extracts at 200 µg/ml and 100 200 µg/ml respectively). Minimum inhibitory concentrations ranged from 50 – 100 µg/ml while minimum bacteriostatic concentrations ranged from 100 – 200 µg/ml. We conclude from our findings that both extracts hold promise in the development of antimicrobial therapeutics.

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Medicinal plants have for thousands of years been used in the management of microbial infections. Plants are natural sources for antimicrobial compounds as they naturally produce a range of antimicrobial compounds to ward off microbial organisms competing with them in their environment (Dhiman *et al.*, 2012; Thawabteh *et al.*, 2019). Several new antibiotics have been produced by pharmacological companies in the past few decades. However, microorganisms have continued to exploit their genetic ability to acquire resistance to therapeutic drugs targeted against them. The high costs and side effects of popular synthetic antibiotics are also topical issues that bother clinicians and drug researchers. This has therefore led scientists to continually screen for and discover newer and potentially better antibiotics from medicinal plants (Tumane *et al.*, 2014; Rajeswari, 2015; Baym *et al.*, 2016; Thawabteh *et al.*, 2019). Random screening of natural plants parts for pharmacologically active compounds has proven to be especially useful in the discovery of antibiotics. Isolation, characterization and chemical synthesis of

identified lead compounds and analogues has proven to be useful globally (Mahesh and Satish, 2008).

*Citrus sinensis* belongs to the *Rutaceae* family. It is native to China but is widely cultivated throughout the world. It is currently the most cultivated and commercialized specie of citrus (Tumane *et al.*, 2014; Yekeen *et al.*, 2014; Favela-Hernández *et al.*, 2016). Fresh citrus fruits are consumed on a large scale and large quantities are processed into juice. Citrus fruit wastes, generated after consumption of the juice include the peels, albedo and seeds. These wastes are a potential source of valuable by products (Oikeh *et al.*, 2013; Yekeen *et al.*, 2014). Citrus peels have been demonstrated to possess several phytochemicals such as phenolics, flavonoids and glycosides. Several studies have reported the antioxidant and antimicrobial effects of citrus juices while citrus peel extracts have also been found to possess good antioxidative potential (Oikeh *et al.*, 2014; Tumane *et al.*, 2014).

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Research into *Citrus sinensis* wastes have focused majorly on the peels and oil extracted from the seed. The non-oil solid residue derived from oil extraction from the seeds is usually not given paid attention to. This study compares the antibacterial activities of oil extracted from *Citrus sinensis* seed as well as the non-oil seed extract. The results in this paper draw more attention to the non-oil extract from the seed as a potential source of bioactive materials with antimicrobial importance.

## MATERIALS AND METHODS

**Raw materials:** Fresh oranges were purchased from New Benin Market in Benin City, Nigeria. The fruits were washed with distilled water and the peels removed with the aid of a sharp knife. A juice extractor was used to extract the juice and the seeds collected. The seeds were pulverized immediately after collection.

**Preparation of plant extract:** The pulverized samples were subjected to Soxhlet extraction for a period of 12 hours with 500 ml of ethanol under room temperature and concentrated using rotary evaporator at low pressure. The top oil layer was separated from the lower solid extract by carefully pouring. The oil and non-oil extract were stored in the refrigerator till required for use.

**Test microorganisms:** Five bacterial strains were used in this study. Two were Gram positive (*Staphylococcus aureus* and *Enterococcus faecalis*) while the other three were Gram negative (*Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella spp*). All microorganisms were obtained from Lahor Research and Diagnostic Laboratories, Benin City, Nigeria.

**Antimicrobial Susceptibility assay:** Test organisms were sub-cultured onto fresh suitable broth medium. Broth cultures were then incubated at 37°C till the turbidity of 0.5 McFarland's standard was obtained. Mueller-Hinton agar was used as bacterial medium. The turbidity of the actively growing broth culture was then adjusted with sterile saline to obtain 0.5 McFarland's standard turbidity ( $10^8$  CFU/ml). This was then used to flood the surface of solid Mueller-Hinton agar plates and then drained dry. Wells of 5 mm in diameter and about 2 cm apart were punched in the culture media with sterile cork borer. The extracts were then used to fill the boreholes. Each plate was kept in the refrigerator at 4 °C for 1 hr before incubating at 37 °C for 24 hr. Zones of inhibition around the wells, measured in millimetres, were used as positive bioactivity.

**Minimum inhibitory concentration (MIC):** The organisms that showed susceptibility to the different solvent extracts were then introduced into the broths containing different concentrations of each extract (Serial dilutions of the extracts corresponding to 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml and 12.5 µg/ml). The tubes were then incubated for 24 hours at 37°C. The MIC was taken as the lowest concentration of the extracts that did not permit any visible growth.

**Minimum bactericidal concentration (MBC):** The tubes that showed no turbidity in the MIC test were taken and a loop-full from each tube was streaked on Mueller Hinton agar. The plates were incubated for 24 hours at 37°C and the absence of growth was observed. The concentration of the extracts that showed no growth was recorded as the MBC.

## RESULTS AND DISCUSSION

The data of plants containing bioactive components is by no means a complete one as several plant species with medicinal value are still yet to be discovered. Thus, screening of plants for a wide array of medicinal properties is an ongoing process. Several plants and plant parts hold promise as rich reservoirs of antimicrobial compounds and thus require sustained pharmacological investigations (Pandey *et al.*, 2011). We have earlier on shown that *Citrus sinensis* seeds contain phytoconstituents such as alkaloids, flavonoids, tannins, saponins, cardiac glycosides and steroids (Oikeh *et al.*, 2013). Several of these phytoconstituents are known to contain a wide spectrum of antimicrobial properties (Owoseni and Ajayi, 2010; Dhiman *et al.*, 2012).

The agar disc diffusion method was used *in vitro* to assay for the antibacterial activity of the *Citrus sinensis* seed oil and extract against five (5) bacterial strains. Tables 1 and 2 show the bacterial growth inhibition of both extracts against the selected bacterial strains. The results show that both *C. sinensis* seed and oil extracts possess varying degrees of antibacterial activities against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Salmonella spp*. *Escherichia coli* was however not susceptible to the *C. sinensis* seed oil as no inhibition of growth was observed. Minimum inhibitory concentration was lowest in the *C. sinensis* seed oil against *Pseudomonas aeruginosa* and *Salmonella spp* (50 µg/ml) with an MBC of 100 µg/ml for these organisms (Table 3). An MIC value of 100 µg/ml was observed for the seed extract against all the bacterial strains tested. Maximum antibacterial activity for the seed extract was observed against *Staphylococcus aureus* with a 12 mm zone of inhibition while the seed oil had Maximum antibacterial activity against

*Salmonella spp* (8 mm zone of inhibition). Measurement of zones of inhibition against Gram-positive and Gram-negative bacteria strains show that the seed extract had larger zones of inhibition than the

seed oil against both Gram-positive organisms (*Staphylococcus aureus* and *Enterococcus faecalis*) at the concentrations studied.

**Table 1:** Zones of inhibition (mm) of some Gram positive bacterial strains at various concentrations of extracts of fresh *Citrus sinensis* seed.

Concentration (µg/ml)	<i>Staphylococcus aureus</i>		<i>Enterococcus faecalis</i>	
	Seed Extract	Seed Oil	Seed Extract	Seed Oil
200	12	4	8	6
100	8	2	4	3
50	-	-	-	-

**Table 2:** Zones of inhibition (mm) of some Gram negative bacterial strains at various concentrations of extract of fresh *Citrus sinensis* seed.

Concentration (µg/ml)	<i>Escherichia coli</i>		<i>Salmonella spp.</i>		<i>Pseudomonas aeruginosa</i>	
	Seed Extract	Seed Oil	Seed Extract	Seed Oil	Seed Extract	Seed Oil
200	2	-	4	8	4	4
100	1	-	2	6	2	2
50	-	-	-	4	-	1

**Table 3:** Minimum inhibitory and bacteriostatic concentrations (µg/ml) of *C. sinensis* seed extracts against microbial strains.

	Gram positive				Gram negative					
	<i>Staphylococcus aureus</i>		<i>Enterococcus faecalis</i>		<i>Pseudomonas aeruginosa</i>		<i>Escherichia coli</i>		<i>Salmonella spp.</i>	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Seed Extract	100	200	100	200	100	200	100	200	100	200
Seed Oil	100	200	100	200	50	100	-	-	50	100

The *Citrus sinensis* seed oil did not show any antibacterial activity against *Escherichia coli* as demonstrated by the lack of any visible zone of inhibition. This is in contrast to the seed extract with 1 and 2 mm zones of inhibition at 100 and 200 µg/ml of the extract respectively. These observed zones of inhibition are however not high enough to conclude that the seed extract may be a good source of antimicrobial agents against *E. coli*.

The antimicrobial activities of the *Citrus sinensis* seed oil and extract observed in this study against different bacterial strains suggests that these extracts may be starting materials for the development of broad-spectrum antimicrobial agents. Variations in the degree of antimicrobial activity of both extracts suggest that both extracts may have diverse bioactive compounds responsible for their antibacterial activities.

Differences in the antimicrobial activities observed may also be attributed to the differences in the nature of both samples. The hydrophobic nature of the oil may have prevented the uniform diffusion of the oil through the agar medium (Hammer *et al.*, 1999). Bearing this in mind, the better antibacterial activity of the oil observed against *Salmonella spp.* suggests that the oil may be a reservoir of potent antibacterial agent against this bacteria.

**Conclusion:** We conclude from our findings that oils extracted from oil-rich plant materials may not be

responsible for all the health benefits ascribed to the plant. This study has provided preliminary *in vitro* data on the antibacterial potential of *Citrus sinensis* seed oil and non-oil extract. Taking into cognizance the wide differences existing in published works as per the antibacterial activities of plant materials *in vitro*; the authors recommend further *in vitro* and *in vivo* studies for the possible identification of compound(s) responsible for the bioactivities.

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